EFFICIENT SYNTHESIS OF RABBIT GLOBIN IN A CELL-FREE SYSTEM FROM WHEAT EMBRYO

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1. Introduction

A cell-free preparation isolated from wheat embryos has been used successfully for the analysis of protein chain initiation with natural mRNA's primarily of plant viral origin [1–4]. A tacit assumption in these studies has been that the ribosome–mRNA attachment reaction with the viral RNA's was equivalent to the reaction with cellular mRNA's. In the present communication we test this assumption by inquiring as to whether rabbit globin mRNA [5] can be translated in the wheat embryo cell-free system. This experiment has the additional significance of providing a more rigorous examination of the question as to whether ribosome systems discriminate between mRNA's. It has been shown in other studies [6–13] that globin mRNA's can be translated *in vitro* in systems from mammalian species that normally do not encounter this mRNA. Wheat embryos are phylogenetically quite distant from mammals. As such, an efficient synthesis of rabbit globin in the wheat system would considerably extend the concept of the lack of ribosome and initiation factor specificity. We report herein that this is indeed the case. Rabbit globin mRNA catalyzes the incorporation of leucine in the wheat embryo cell-free system at a rate equivalent to that obtained with the most active heterologous mammalian systems. The products of the reaction are examined by a number of criteria and shown to be exclusively the α and β chains of rabbit globin.

2. Materials and methods

2.1. *Amino acid incorporation reaction*

A 0.10 ml incubation mixture contained: 25 mM Tris–acetate pH 8.0, 50 mM KCl, 3.6 mM $Mg(Ac)_2$, 2.2 mM dithiothreitol, 1 mM ATP, 0.25 mM GTP, 8 mM creatine phosphate, 6 μg creatine phosphate kinase, 3 μg wheat germ tRNA [14], 0.125 μCi [^{14}C]-leucine (0.4 nmole), a mixture of the remaining 19 amino acids to 0.06 mM, 2 μg (0.055 A_{260} units) of rabbit globin mRNA and 30 μl dialyzed wheat embryo S-23 (the post-mitochondrial supernatant obtained by centrifuging a wheat embryo homogenate for 10 min at 23,000 *g* (see [14]). The incubation was carried out at 30°C for 1 hr.

Rabbit globin mRNA and [^{3}H]leucine labeled rabbit haemoglobin were generously made available by Dr. Ronald Crystal. The procedure for preparing the globin mRNA has been published [15].

2.2. *Analysis of the products*

Products containing [^{14}C]leucine from the *in vitro* incubation were supplemented with rabbit haemoglobin labeled *in vivo* with [^{3}H]leucine, and the combined mixture was brought to 10 mM EDTA and 6.25 mM unlabeled leucine. 0.1 mg/ml pancreatic RNAase was added and incubation was continued for 10 min at 37°C.

For acrylamide gel electrophoresis, the RNAase treated products were precipitated and washed follow-

ing the procedure of Aviv and Leder [12]. The final precipitate was dissolved in 8 M urea, 1% sodium dodecyl sulphate, 2.5% (v/v) 2-mercaptoethanol, 0.01 M sodium phosphate pH 7.0. The sample was heated at 100°C for 2 min and applied to a 0.6 × 10 cm 7.5% acrylamide gel [16] containing 6 M urea, 0.1% sodium dodecyl sulphate and 0.1 M sodium phosphate pH 7.0. A current of 8 mA/gel was applied for 6 hr allowing the marker to migrate 9 cm. Gels were cut into 1.0 mm pieces, placed in vials containing 0.5 ml H_2O_2 and stored overnight at 50°C. Each sample was counted with 6.0 ml Aquasol (New England Nuclear Inc.).

For analysis by CMC chromatography the RNAase treated products were mixed with 25 mg of unlabeled haemoglobin and the heme extraction was performed as described by Crystal et al. [15]. The final pellet was dissolved in 2.0 ml of 0.2 M formic acid, 0.02 M pyridine, and applied to a column of Whatman CM-52 (1.1 × 12 cm) which had been previously equilibrated with the same solution. The column was washed with 10 ml of the same solution and eluted with a linear gradient consisting of 125 ml each of 0.2 M formic acid, 0.02 M pyridine and 2.0 M formic acid, 0.2 M pyridine [17]. Fractions of 2.8 ml were collected and 0.2 ml aliquots were applied to glass fiber filters which were then counted in a toluene scintillator (5 g PPO and 300 mg dimethyl POPOP per ℓ).

For peptide analysis the incubation mixture was increased 4-fold and the RNAase treated products were processed as described for the electrophoretic analysis. The final pellet was dissolved in 4.0 ml 0.1 M NH_4HCO_3 pH 8.0, containing 400 μg TPCK-trypsin and 40 μg chloramphenicol. After overnight incubation at 37°C, an additional 400 μg of TPCK-trypsin was added and incubation was continued for 3 hr. The NH_4HCO_3 was removed by repeated evaporation from water and the digested material was dissolved in 3.0 ml of 0.05 M pyridine acetate pH 2.6. This solution was then applied to a cation exchange column (1×13 cm type DC-2; Durrum Chemical Corporation, Palo Alto, Calif.). Peptide elution was carried out with a gradient consisting of 120 ml of 0.05 M pyridine acetate pH 2.6 and 120 ml 1.2 M pyridine acetate pH 5.0 at a flow rate of 10.0 ml per hour. Fractions of 2.0 ml were collected and aliquots of 0.2 ml were applied to glass fiber filters which then were dried and counted for radioactivity.

3. Results

Purified globin mRNA stimulates the incorporation of [^{14}C]leucine into protein in the wheat embryo cell-free system better than 20-fold (table 1). The KCl optimum for the incorporation reaciton is considerably below that reported for other eukaryotic mammalian systems responding to globin mRNA [19]. Rather it corresponds to that previously determined for TMV-RNA dependent amino acid incorporation in the wheat embryo system [20].

The products synthesized in the *in vitro* reaction in response to the addition of rabbit globin mRNA were analyzed by comparison with authentic globin by three methods: SDS-urea acrylamide gel electrophoresis (fig. 1), CMC chromatography (fig. 2), and tryptic peptide analysis (fig. 3).

The migration of the SDS-urea acrylamide gels (fig. 1), in spite of a small quantity of aggregation (fractions 40–45), shows a single peak containing both the authentic globin labeled with [^{3}H]leucine and the *in vitro* product containing [^{14}C]leucine. Fig. 2 demonstrates that the *in vitro* wheat embryo system synthesizes both the α and β chains of rabbit globin. Summing the radioactivity of the respective peaks of the [^{14}C]-labeled *in vitro* product we obtain 1,581 cpm for the α chain and 1,613 cpm for the β chain. A similar determination of the [^{3}H]leucine-labeled *in vivo* product gives 12,020 cpm for the α chain and 12,161 cpm for the β chain. Thus the two chains are synthesized in a ratio of about 1:1.

Table 1
Incorporation of [^{14}C]leucine into protein by wheat embryo S-23 in response to added rabbit globin mRNA.

KCl concentration (mM)	[^{14}C]leucine incorporated (pmoles)
50	181.6
75	82.2
100	1.4
50*	8.3

The incubation was carried out as described in Methods. After 1 hr at 30°C, hot TCA-insoluble radioactivity was determined as previously described [18]. In the incubation noted by the asterisk, the globin mRNA was omitted. The specific activity of the [^{14}C]leucine was 311 mCi/mM. With a counting efficiency of 50%, this calculates to 340 cpm per pmole.

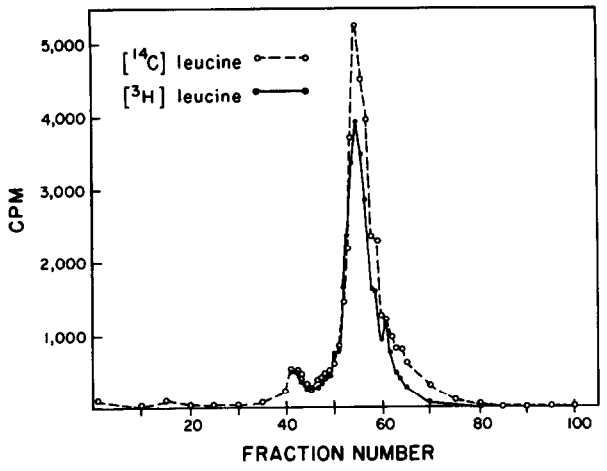

Fig. 1. Acrylamide SDS-urea gel electrophoresis of products synthesized in the wheat embryo cell free system in response to rabbit globin mRNA. The products of a single incubation containing 72,000 cpm of ^{14}C were mixed with [^{3}H]-labeled haemoglobin (70 μg containing 55,000 cpm) and prepared for electrophoresis as described in Methods.

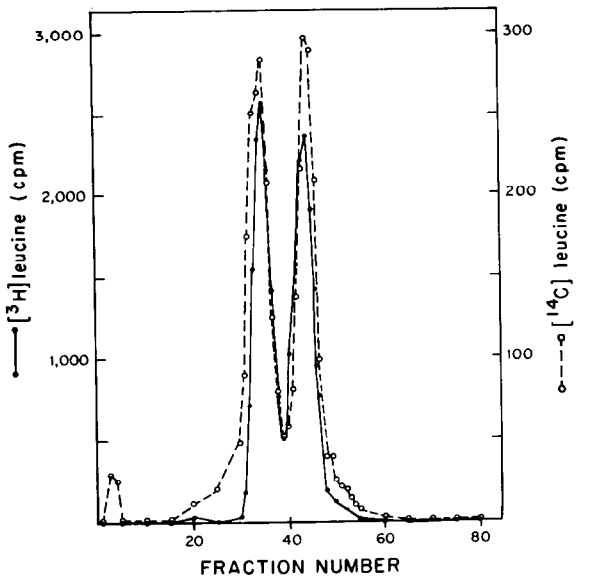

Fig. 2. Chromatography of globin synthesized *in vivo* and *in vitro* on CM-cellulose column. A sample containing 600,000 cpm of haemoglobin (750 μg) labeled *in vivo* with [^{3}H]leucine, 25 mg unlabeled haemoglobin and 80,000 cpm of [^{14}C]leucine labeled product was anlyzed by CMC chromatography as described in Methods.

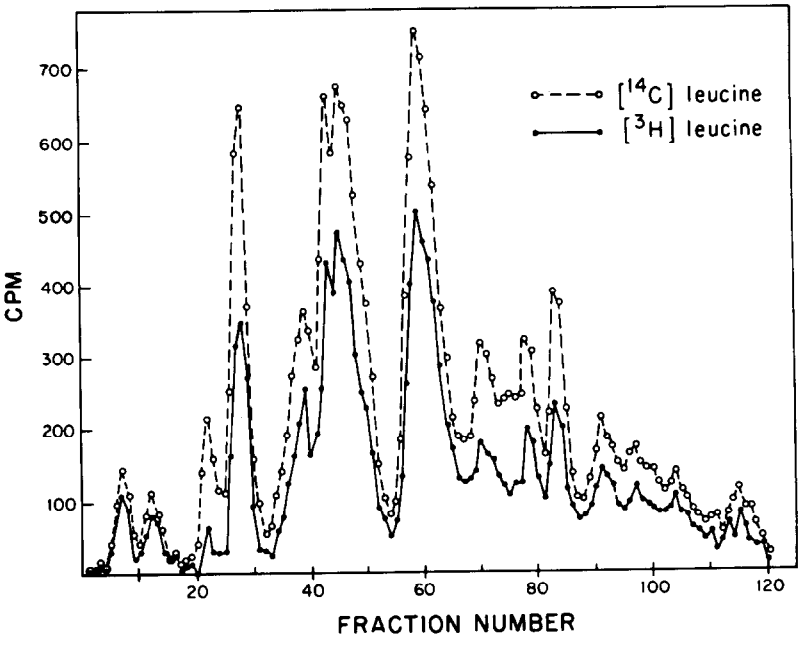

Fig. 3. Tryptic peptides analysis of [^{14}C]leucine labeled products synthesized *in vitro* by the cell-free wheat embryo system in response to rabbit globin mRNA. [^{3}H]haemoglobin containing 200,000 cpm in 250 μg was added to the products of a 4-fold increased incubation mixture. The latter contained 306,000 cpm of ^{14}C. The sample was treated with trypsin and the tryptic peptides were analyzed as described in Methods.

Evidence establishing the authenticity of the globin synthesized by the wheat embryo system is obtained by the analysis of the tryptic peptides of the *in vitro* product. The α and β chains of rabbit haemoglobin should be digested into 17 leucine-containing peptides by trypsin treatment [21]. As seen in fig. 3 there are indeed 17 [^{14}C]leucine-labeled tryptic peptides derived from the *in vitro* product, each peptide corresponding to a [^{3}H]leucine-labeled peptide obtained from the carrier *in vivo* labeled globin. Finally, the ratio of radioactivity in the different peptides of the *in vitro* synthesized product corresponds closely to that of the globin synthesized *in vivo*.

4. Discussion

Translation of rabbit globin mRNA in the wheat embryo system is a very efficient reaction and occurs with a high degree of accuracy. As a matter of routine, 19 unlabeled amino acids are added in the cell-free system. There is, however, essentially no requirement for this supplement, indicating an endogenous supply of unlabeled amino acids at least equivalent to the amount of radioactive amino acid incorporated. In addition, in experiments testing the effect of the level of globin mRNA on amino acid incorporation, we have found almost the same extent of reaction for 0.5 μg (2.5 pmoles) as that obtained with 2 μg. These considerations allow the conclusion that the wheat embryo system is capable of synthesizing at least 8 chains of globin (assuming 17 leucine residues per globin chain see [21]) per chain of mRNA per hour, a rate equivalent to that obtained in the most active heterologous mammalian cell-free systems [11, 22].

The accuracy of globin translation in the wheat embryo system is particularly evident from the tryptic peptide analysis (fig. 3) demonstrating an almost perfect equivalence between the products made *in vivo* and *in vitro*. The reason for this high degree of correlation is that the wheat embryo cell-free preparation contains only messenger-free ribosomes. As such, there is little background formation of other *in vitro* products.

Previous analyses of the chain content of rabbit globin synthesized in heterologous systems have shown a 1.5-fold excess of α to β chain with a mouse liver preparation [10] and a similar excess in reverse (i.e., β over α) with Krebs ascites cells [8]. The results with the wheat embryo system seem to parallel the situation *in vivo* in rabbit reticulocytes, with the two globin chains made in a ratio of about 1:1. The experiments of Lodish [23] have indicated that rabbit reticulocyte polyribosomes have about 1.5 times as much α-chain mRNA as β-chain mRNA suggesting that the *in vivo* equivalence of products is due to more efficient initiation on the β-mRNA. If this is so, then the results of the chain analyses in the wheat embryo system indicate that this system preserves *in vitro* the discrimination otherwise seen only *in vivo*.

The demonstration that globin mRNA can be efficiently translated in a plant cell-free system, together with the earlier observations that globin mRNA can be translated *in vitro* in a number of heterologous mammalian systems [6–13] and *in vivo* in frog oocytes [24], clearly establish a lack of specificity of mRNA recognition with regard to the translational machinery of the ribosomes. Yet several lines of evidence indicate that there exists a translational control mechanism operative at the level of mRNA–ribosome interaction. The studies of Groner et al. [25] and Lee-Huang and Ochoa [26] have shown the existence of protein factors that prevent ribosome attachment at specific cistronic mRNA initiation sites, thereby providing a system for effective mRNA selection. More recently, Lebleu et al. [22] have reported that translation of mengo virus RNA in an ascites cell-free system is specifically stimulated by L-cell factors under conditions that reticulocyte factors are inactive. In studies with the wheat embryo system, it has been observed that MS2 and Qβ RNA are completely inactive in catalyzing amino acid incorporation [4, 27]. Under the same conditions, both STNV-RNA [4] and, as shown here, globin mRNA are translated with a high degree of accuracy. Furthermore, *in vitro* translation of TMV-RNA in the wheat embryo system results in a wide gamut of products [28] despite the fact that *in vivo*, in infected tobacco plants, the only detectable product synthesized as a consequence of viral infection is the TMV coat protein [29]. Cumulatively, these observations indicate that in eukaryotic cells, as in bacteria, there must exist at least to some extent mechanisms for selective translation of specific mRNA's.

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References

[1] A. Marcus, D.P. Weeks, J. Leis and E.B. Keller, Proc. Natl. Acad. Sci. U.S. 67 (1970) 1681.
[2] S.N. Seal, J.D. Bewley and A. Marcus, J. Biol. Chem. 247 (1972) 2592.
[3] D.P. Weeks, D.P.S. Verma, S.N. Seal and A. Marcus, Nature 236 (1972) 167.
[4] W.H. Klein, C. Nolan, J.M. Lazar and J.M. Clark, Jr., Biochemistry 11 (1972) 2009.
[5] H. Chantrenne, A. Burny and G. Marbaix, Prog. Nucleic Acid Res. Mol. Biol. 7 (1967) 173.
[6] R.E. Lockard and J.B. Lingrel, Biochem. Biophys. Res. Commun. 37 (1969) 204.
[7] M.B. Mathews, M. Osborn and J.B. Lingrel, Nature New Biol. 233 (1971) 206.
[8] D. Housman, R. Pemberton and R. Tabor, Proc. Natl. Acad. Sci. U.S. 68 (1971) 2716.
[9] P.M. Prichard, D.J. Piciano, D.G. Laycock and W.F. Anderson, Proc. Natl. Acad. Sci. U.S. 68 (1971) 2752.
[10] J. Lampson, M.B. Mathews, M. Osborn and A.F. Borghetti, Biochemistry 11 (1972) 3636.
[11] S. Metafora, M. Terada, L.W. Dow, P.A. Marks and A. Bank, Proc. Natl. Acad. Sci. U.S. 69 (1972) 1299.
[12] H. Aviv and P. Leder, Proc. Natl. Acad. Sci. U.S. 69 (1972) 1408.
[13] A.J.M. Berns, R.A. de Abreu, M. van Kracukamp, E.L. Benedetti and H. Bloemendal, FEBS Letters 18 (1971) 159.
[14] A. Marcus, D. Efron and D.P. Weeks, in: Methods in enzymology, Part E, eds. L. Grossman and K. Moldave (1973) in press.
[15] R.G. Crystal, A.W. Nienhius, N.A. Elson and W.F. Anderson, J. Biol. Chem. 247 (1972) 5357.
[16] J.V. Maizel, Jr., in: Methods in virology, Vol. V, eds. K. Maramorosch and H. Koprowski (1971) p. 179.
[17] H.M. Dintzis, Proc. Natl. Acad. Sci. U.S. 47 (1961) 628.
[18] A. Marcus, J. Feeley and T. Volcani, Plant Physiol. 41 (1966) 1167.
[19] M.B. Mathews, Biochim. Biophys. Acta 272 (1972) 108.
[20] A. Marcus, J. Biol. Chem. 245 (1970) 955.
[21] M.O. Dayhoff and R.V. Eck, Atlas of protein sequence and structure (1967–68) pp. 128, 142.
[22] B. Lebleu, U. Nudel, E. Falcoff, C. Prives and M. Revel, FEBS Letters 25 (1972) 97.
[23] H.F. Lodish, J. Biol. Chem. 246 (1971) 7131.
[24] C.D. Lane, G. Marbaix and J.B. Gurdon, J. Mol. Biol. 61 (1971) 73.
[25] Y. Groner, Y. Pollack, H. Berissi and M. Revel, FEBS Letters 21 (1972) 223.
[26] S. Lee-Huang and S. Ochoa, Nature New Biol. 234 (1971) 236.
[27] A. Marcus, in: Protein biosynthesis in nonbacterial systems, Vol. 2, eds. A.I. Laskin and J.A. Last (1972) p. 127.
[28] D. Efron and A. Marcus, Virology (1973) in press.
[29] B. Singer, Virology 46 (1971) 247.